Effect of short-term cold exposure on skeletal muscle protein breakdown in rats

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Manfredi LH, Zanon NM, Garófalo MA, Navegantes LC, Kettelhut IC. Effect of short-term cold exposure on skeletal muscle protein breakdown in rats. J Appl Physiol 115: 1496–1505, 2013. First published August 1, 2013; doi:10.1152/japplphysiol.00474.2013.—Although it is well established that carbohydrate and lipid metabolism are profoundly altered by cold stress, the effects of short-term cold exposure on protein metabolism in skeletal muscle are still poorly understood. Because cold acclimation requires that an organism adjust its metabolic flux, and muscle amino acids may be an important energy source for heat production, we hypothesize that muscle proteolysis is increased and protein synthesis is decreased under such a stress condition. Herein, cold exposure for 24 h decreased rates of protein synthesis and increased overall proteolysis in both soleus and extensor digitorum longus (EDL) muscles, but it did not affect muscle weight. An increase in proteolysis was accompanied by hyperactivity of the ubiquitin-proteasome system (UPS) in both soleus and EDL, and Ca2+-dependent proteolysis in EDL. Furthermore, muscles of rats exposed to cold showed increased mRNA and protein levels of atrogin-1 and muscle RING finger enzyme-1 (MuRF1). Additionally, cold stress reduced phosphorylation of Akt and Forkhead box class O1 (FoxO1), a well-known effect that increases FoxO translocation to the nucleus and leads to activation of proteolysis. Plasma insulin levels were lower, whereas catecholamines, corticosterone, and thyroid hormones were higher in cold-exposed rats compared with control rats. The present data provide the first direct evidence that short-term cold exposure for 24 h decreases rates of protein synthesis and increases the UPS and Ca2+-dependent proteolytic processes, and increases expression of atrogin-1 and MuRF1 in skeletal muscles of young rats. The activation of atrophy induced by acute cold stress seems to be mediated at least in part through the inactivation of Akt/FoxO signaling and activation of AMP-activated protein kinase.

cold; protein degradation; protein synthesis; atrogin-1; muscle RING finger enzyme-1

HOMEOThERMIC ANIMALS EXPOSED to low environmental temperatures activate heat-saving mechanisms that lead to changes in their basal metabolism and other physiological systems. The hypothalamic-pituitary-thyroid axis and the sympathoadrenal system are activated, and the new hormonal scenario induces adjustments in the rates of metabolic processes to provide support to life in animals exposed to cold (11).

The increase in catecholamines and thyroid hormones in cold-exposed animals promote an increase in heat production through activation of brown adipose tissue (3), lipolysis in white adipose tissue, glycogenolysis in liver and skeletal muscle, and gluconeogenesis in liver (14, 45). These changes in lipid and carbohydrate metabolism are crucial for providing fuel to tissues that can produce heat, especially the brown adipose tissue and skeletal muscle (50). Although proteins may account for less than 15% as fuel when rats are exposed to cold (44), little is known about the adaptive changes that occur in skeletal muscle protein metabolism during cold exposure.

Skeletal muscle mass is maintained by a precise balance between protein synthesis and protein breakdown (19, 21). It has been shown that long-term exposure to cold leads to a reduction in the rate of muscle protein synthesis in different species, including calves and rats, which can explain, at least in part, the decreased protein mass during cold acclimation (34, 36). In another study, McAllister et al. (26) measured the fractional rates of degradation in vivo in skeletal muscle in weaning rats over 5 and 20 days, and concluded that degradation did not differ between cold-exposed and normal rats. Despite this evidence, the molecular mechanisms underlying either the reduction in protein synthesis or participation of different proteolytic systems in short-term or long-term cold exposure have never been addressed.

The three main proteolytic processes involved in the control of muscle protein metabolism in mammals are lysosomal, Ca2+-dependent, and the ubiquitin (Ub)-proteasome system (UPS). The acid hydrolases in lysosomes degrade the majority of extracellular and membrane proteins taken up by endocytosis and by cytoplasmic proteins and organelles from autophagy (9, 37). The Ca2+-dependent process contains at least two ubiquitous enzymes: calpain 1, or µ-calpain (low Ca2+-requiring form); and calpain 2, or m-calpain (high Ca2+-requiring form). Calpains are heterodimers constituted by an 80-kDa catalytic subunit and a 28-kDa regulatory subunit, and thyroid hormones were higher in cold-exposed rats compared with control rats. The present data provide the first direct evidence that short-term cold exposure for 24 h decreases rates of protein synthesis and increases the UPS and Ca2+-dependent proteolytic processes, and increases expression of atrogin-1 and MuRF1 in skeletal muscles of young rats. The activation of atrophy induced by acute cold stress seems to be mediated at least in part through the inactivation of Akt/FoxO signaling and activation of AMP-activated protein kinase.

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Conversely, decreases in this signaling pathway in skeletal muscle during physiological conditions of muscle atrophy such as starvation and muscle disuse are believed to contribute to FoxO and atrogen activation. Because cold exposure is a physiological condition in which a set of hormonal adjustments occur to provide energy substrates for heat production, and some changes such as high glucocorticoids and T3 levels and low insulin levels, negatively interfere with Akt signaling (17, 28, 49), we hypothesized that cold exposure would have negative effects on muscle protein balance by inhibiting rates of protein synthesis and by stimulating protein breakdown. Thus, the purpose of the current study was to examine the impact of short-term cold exposure (24 h at 4°C) on muscle protein metabolism in juvenile rats. Specifically, we examined the rate of protein synthesis, overall proteolysis, activity of three proteolytic systems (lysosomal, UPS, and Ca2+-dependent), gene expression, and protein levels involved in the process of ubiquitination as well as Akt/FoxO signaling and AMP-activated protein kinase (AMPK) in skeletal muscle.

**MATERIAL AND METHODS**

**Animals and treatment.** Because the incubation procedure required intact muscles of a sufficient thinness to allow for the adequate diffusion of metabolites and oxygen, 4-wk-old male Wistar rats (~80 g) were used in all experiments. Animals were housed in a room with a 12:12-h light/dark cycle (starting at 6 A.M.) at 25 ± 2°C and were given free access to water and lab chow diet (CR1, Nuvital, Brazil) for at least 2 days before the experiments. Because preliminary experiments showed that animals exposed to cold during the first 24 h eat less or do not eat compared with controls, the food was removed at the beginning of the exposure. Animals were maintained in individual cages at 4°C ± 1°C for 3, 6, 12, or 24 h, and the controls were kept at 25°C. The total number of animals used was ~60. For overall proteolysis and synthesis assays we used six or seven rats from each group and isolated soleus or extensor digitorum longus (EDL) muscles from one leg. Contralateral muscles were removed and stored at ~80°C for Western blot or RT-PCR experiments. For measurement of proteolytic system activities we used both muscles (left and right) from the same animal. An additional group of six animals per group was submitted to 4 days of cold exposure with free access to food and water. All rats were killed by cervical dislocation with the exception of a separate group of animals that was killed by decapitation for metabolic and hormone measurements. All experiments and protocols were performed in accordance with the ethical principles for animal research adopted by the Brazilian College of Animal Experimentation and were approved by the Ethical Commission of Ethics in Animal Research (No. 130/2009) of the School of Medicine of Ribeirão Preto, University of São Paulo, Brazil.

**Incubation procedure.** Soleus and EDL muscles were rapidly dissected, avoiding any damage to the muscles. Muscles were maintained at resting length by pinching their tendons in aluminum or plastic supports. They were incubated at 37°C in Krebs-Ringer bicarbonate buffer pH 7.4, equilibrated with 95% O2 and 5% CO2 containing glucose (5 mM), and in the presence of cycloheximide (0.5 mM) to prevent protein synthesis and reincorporation of tyrosine back into proteins. Tissues were preincubated for 1 h and then incubated for 2 h in fresh medium of identical composition.

**Protein degradation measurement.** Rates of overall proteolysis and of different proteolytic systems were determined by measuring the rate of tyrosine release in the incubation medium. Because muscles cannot synthesize or degrade tyrosine, tyrosine release reflects the rate of protein breakdown. Tyrosine was assayed by a fluorimetric method as previously described (46).

For measurement of lysosomal proteolysis, muscles from one limb were incubated in the absence of methylamine, insulin, and branched-chain amino acids, a condition in which the lysosomal system is activated. Contralateral muscles were incubated in the presence of insulin (1 IU/ml), leucine (170 μM), isoleucine (100 μM), valine (200 μM), and methylamine (10 mM), a weak base that increases intralysosomal pH and inhibits lysosomal proteolysis. For measurement of Ca2+-dependent proteolytic activity, muscles from one limb were incubated in the presence of Ca2+ and inhibitors of the aforementioned lysosomal system. Contralateral muscles were incubated in a Ca2+-free medium that contained lysosomal inhibitors and cysteine-protease inhibitors (50 μM E64 and 25 μM leupeptin). For measurement of UPS activity, muscles from one limb were incubated under conditions that prevent activation of the lysosomal and Ca2+-dependent proteolytic systems as described above. In addition, muscles from the contralateral limb were incubated with the proteasome inhibitor MG132 (20 μM). UPS, lysosomal, and Ca2+-dependent proteolytic activities were calculated from the difference in tyrosine release between the left and right muscles.

**Measurement of rates of protein synthesis.** To measure rates of protein synthesis in soleus and EDL muscles, L-[U-14C] tyrosine (0.05 μCi/ml) was added in Krebs-Ringer bicarbonate buffer without cycloheximide after preincubation, and muscles were incubated for 2 h. Afterward, the muscles were used to measure the specific activity of acid-soluble tyrosine (intracellular tyrosine pool) by measuring the radioactivity and concentration of tyrosine by a previously described method (46). After measurement of the radioactivity incorporated into protein of the same muscle, the rate of synthesis was calculated using the specific activity of the intracellular pool of tyrosine, assuming that there was no recycling of the label during the incubation period (12, 42).

**Western blotting analysis.** Soleus and EDL muscles were immediately frozen in liquid nitrogen after exciting them from the animal. Muscles were homogenized in 50 mM Tris-HCl buffer (pH 7.4) at 4°C containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM sodium orthovanadate, 5 μg/ml apro- tinin, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 14,000 g at 4°C for 30 min, retaining the supernatant, and protein content was determined using BSA as a standard (25). An equal volume of sample buffer (20% glycerol, 125 mM Tris-HCl, 4% SDS, 100 mM dithiothreitol, 0.02% bromophenol blue, pH 6.8) was added to the supernatant and the mixture was boiled. Fifty micrograms of protein was subjected to SDS-PAGE analysis on 10%, 15%, or 12% acrylamide gels depending on the size of the protein. Control and experimental samples were always run on the same gel. Gels were electroblotted onto nitrocellulose membranes (43) and blotted with anti-μ-calpain and anti-m-calpain, anti-calpastatin, anti-α-fodrin, anti-p-[Ser57]-Akt, anti-Akt, anti-p[Thr22]-AMPK, anti-AMPK, anti-p-[Thr32]-FoxO3a, anti-p-[Ser323]-FoxO3a, anti-FoxO3a, anti-p-[Ser202]-FoxO1, anti-p-[Thr21]-FoxO1, anti-FoxO1, anti-atrogin-1, anti-MuRF1, anti-p[Thr70]4E-BP1, anti-4E-BP1, anti-p-[Ser379]-eIF4E, anti-eIF4E, and anti-α-tubulin. Primary antibodies were detected by peroxidase-conjugated secondary antibody and visualized via enhanced chemiluminescence reagents and detected with a Molecular Imager (ChemiDoc XRS+, BioRad). Band intensities were quantified using Image Lab (BioRad).

**Calpain activity.** Calpains cleaves the 280-kDa neuroskeletal protein α-fodrin (also referred to as α-spectrin in erythrocytes) into a 150-kDa fragment, which is subsequently and sequentially cleaved to a 145-kDa residue by calpains (48). Thus the α-fodrin immunoblot was used to indirectly but reliably estimate the activity of calpains (1, 27, 33).

**Quantitative polymerase chain reaction.** RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) from soleus and EDL muscles. Reverse transcription into cDNA was performed using 2 μg of total cellular RNA, 20 pmol oligo(dT) primer (Invitrogen), and reverse transcriptase (Advantage ImProm-II; Promega, Madison, WI).
time PCR was carried out on a sequence detection system (ABI-7000; Applied Biosystems, Foster City, C) using a SuperScript III Platinum SYBR Green One-Step RTqPCR Kit with ROX (Invitrogen). The following rat primers were used: atrogin-1 (forward, 5'-GCA-GAGAGCTGGCAAGTC-3'; reverse, 5'-CAGGTCCGTGATCG-AG-3'); MuRF1 (forward, 5'-TCGACATCTACAAGCAGGAA-3'; reverse, 5'-CTGTCCTGGAGAGTGCTTT-3'); calpastatin (forward, 5'-GTCATCACAGGACCTTTCCA-GAGAC-3'; reverse, 5'-CGTGAAAT-CAGATGCAAGGCA-3'); μ-calpain (forward, 5'-CTGAGGGTCA- TAGACCCAGCTTCGTTCTCCTC-3'); Rpl39 (forward, 5'-GATGGAGGAC-3'; reverse, 5'-TAGACCCAGCTTTCCA-GAGAC-3'). Transcript levels of the target gene were normalized with Rpl39. This gene was used as a control

Metabolic and hormonal measurements. In a separate group of animals, blood was collected by decapitation and heparinized. Plasma glucose concentration was determined by a glucose oxidase method (Glucox 500; Doles, Goiânia, Brazil). Plasma catecholamines were measured as previously described (13) using HPLC (LC-7A; Shimadzu Instruments) with a 5-μm Spherisorb ODS-2 reversed-phase column (Sigma-Aldrich). Insulin was measured by radioimmunoassay using a commercial kit (DPC-MEDLAB, São Paulo, Brazil) and corticosterone was evaluated according to the method described by Elias et al. (10). Thyroid hormones were measured following the method described by Dare et al. (8). Statistical methods. The effects of cold over time were tested using a two-way ANOVA. When time was not a variable presented in the experiment, the groups were analyzed using a Student’s nonpaired t-test (cold vs. control). To estimate activity of the proteolytic systems, we used a paired t-test to compare the two muscles (left and right) from the same animal. Variability of data is expressed as means ± SE. Differences were considered statistically significant at P < 0.05.

RESULTS

Effect of cold exposure on metabolic and hormonal parameters. As shown in Table 1, exposure to cold (4°C) for 24 h increased by 20% fasting blood glucose levels and plasma levels of corticosterone (100–170%) at all experimental periods. Plasma levels of norepinephrine increased 31% only after 3 h, whereas epinephrine increased after 6 and 12 h, and decreased to control values after 24 h of exposure to cold (46% and 31%, respectively). Exposure to cold for 24 h also increased plasma levels of free T3 (7.13 ± 0.047 pg/ml in controls vs. 5.24 ± 0.047 pg/ml in cold-exposed animals), T4 (1.60 ± 0.09 pg/ml vs. 1.04 ± 0.05 pg/ml in controls and cold-exposed animals, respectively), and total T4 (4.01 ± 0.29 pg/ml vs. 2.62 ± 0.2 pg/ml in controls and cold-exposed animals, respectively); and decreased (30%) plasma levels of insulin (26.6 ± 2 μIU/ml vs. 38.9 ± 3 μIU/ml in controls). After 4 days of cold exposure, corticosterone levels were not different between groups and plasma insulin was still lower in animals exposed to cold compared with controls (Table 2).

Effect of cold exposure on rates of overall protein degradation and protein synthesis. To study whether cold exposure could promote any alteration in skeletal muscle protein degradation, a time-dependent curve of overall proteolysis was established. As shown in Fig. 1A, soleus muscle from rats

Table 1. Hormonal and metabolic parameters from rats exposed to different periods to cold

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Cold</th>
<th>Control</th>
<th>Cold</th>
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</thead>
<tbody>
<tr>
<td>Glycemia, mg/dl</td>
<td>132 ± 2</td>
<td>159 ± 3*</td>
<td>130 ± 4</td>
<td>143 ± 3*</td>
</tr>
<tr>
<td>Adrenaline, ng/ml</td>
<td>8.3 ± 1</td>
<td>7.2 ± 0.7</td>
<td>8.4 ± 0.7</td>
<td>12.2 ± 1*</td>
</tr>
<tr>
<td>Noradrenaline, ng/ml</td>
<td>4.7 ± 0.3</td>
<td>6.14 ± 0.4*</td>
<td>4.4 ± 0.3</td>
<td>4.78 ± 0.4</td>
</tr>
<tr>
<td>Corticosterone, μg/dl</td>
<td>7.7 ± 0.2</td>
<td>19.7 ± 0.9*</td>
<td>7.7 ± 0.7</td>
<td>19.1 ± 0.5*</td>
</tr>
</tbody>
</table>

Values are means ± SE of 6–8 animals; *P < 0.05 cold vs. control.

Table 2. Hormonal and metabolic parameters from rats exposed to 4 days of cold

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Cold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycemia, mg/dl</td>
<td>130 ± 4</td>
<td>147 ± 6*</td>
</tr>
<tr>
<td>Insulin, μIU/ml</td>
<td>38.9 ± 3</td>
<td>19.1 ± 3.03*</td>
</tr>
<tr>
<td>Corticosterone, μg/dl</td>
<td>5.54 ± 0.75</td>
<td>6.10 ± 0.72</td>
</tr>
<tr>
<td>Free fatty acids, μmol/ml</td>
<td>0.405 ± 0.032</td>
<td>0.557 ± 0.048*</td>
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Values are means ± SE of 6–8 animals; *P < 0.05 cold vs. control.

Fig. 1. Overall proteolysis in soleus (A) and extensor digitorum longus (EDL) (B) muscles was measured 3, 6, 12, and 24 h after rats were submitted to cold. Values are presented as means ± SE of 7 muscles; *P < 0.05 vs. control.
Cold-exposed rats exhibited an increase in overall proteolysis (nmol·mg\(^{-1}\)·2 h\(^{-1}\) of tyrosine release) after 6, 12, and 24 h (0.463 \(\pm\) 0.018 vs. 0.370 \(\pm\) 0.013, 0.543 \(\pm\) 0.015 vs. 0.441 \(\pm\) 0.016, and 0.488 \(\pm\) 0.022 vs. 0.358 \(\pm\) 0.007 in cold-exposed vs. controls, respectively). In EDL muscle (Fig. 1B), exposure to cold increased overall proteolysis after 12 and 24 h of cold exposure (0.413 \(\pm\) 0.025 vs. 0.331 \(\pm\) 0.021 and 0.423 \(\pm\) 0.028 vs. 0.310 \(\pm\) 0.019; cold-exposed vs. controls, respectively).

Rates of muscle protein synthesis (nmol·mg\(^{-1}\)·2 h\(^{-1}\) of tyrosine incorporated) decreased about 20% in both soleus (0.478 \(\pm\) 0.019 vs. 0.578 \(\pm\) 0.012; cold-exposed vs. controls) and EDL (0.254 \(\pm\) 0.008 vs. 0.320 \(\pm\) 0.020; cold-exposed vs. controls) muscles from rats exposed to cold for 24 h (Fig. 2A). Although these animals showed an increase in overall proteolysis and a decrease in rate of protein synthesis, they did not exhibit loss of muscle mass (mg per 100 g of body wt) either in soleus (62.3 \(\pm\) 1.6 vs. 61.2 \(\pm\) 1.4; cold-exposed vs. controls) or EDL (47.3 \(\pm\) 0.9 vs. 47.8 \(\pm\) 0.7; cold-exposed vs. controls). However, after 4 days of cold exposure, soleus muscles lost \(\sim\)15% of muscle mass (44.4 \(\pm\) 1.3 vs. 52.3 \(\pm\) 0.9 for controls).

Similar effects were observed in tibialis and gastrocnemius muscles (Fig. 3B). There was a tendency toward decreased muscle mass in EDL from animals exposed to cold for 4 days (38.9 \(\pm\) 0.9 vs. 43.4 \(\pm\) 1.9 for controls), but this did not reach statistical significance.

**Effect of cold exposure on different proteolytic pathways.** Skeletal muscle proteolytic pathways in rats exposed to cold for 24 h varied according to the type of muscle. The activity of the lysosomal proteolytic pathway did not differ significantly in either soleus or EDL muscles from rats exposed to cold for 24 h (Fig. 4, A and B). However, UPS activity was increased in soleus (50%) and EDL (60%) muscles (Fig. 4, A and B). During exposure to cold, the activity of the Ca\(^{2+}\)-dependent proteolytic system was not altered in soleus, but it was markedly increased (3-fold) in EDL muscles (Fig. 4, A and B).

**Effect of cold exposure on expression of Ub-ligases, Akt/ FoxO signaling and AMPK.** To further investigate the mechanisms underlying the cold-induced activation of the UPS, expression of the Ub-ligases atrogin-1 and MuRF-1 was assessed. Also, the signaling pathways involved in the catabolic effect of cold stress on protein metabolism; the phosphorylation status of Akt, FoxO, 4E-binding protein 1 (4E-BP1), and AMP-activated protein kinase (AMPK) was assessed in muscles from control rats and those exposed to cold for 24 h. As shown in Fig. 5, cold exposure increased mRNA levels and protein content of atrogin-1 and MuRF1, an effect that was associated with low levels of phosphorylation status of AktSer in both soleus and EDL muscles (Fig. 6A). Furthermore, cold stress decreased the phosphorylation levels of FoxO3aThr32 and FoxO1Thr24 in soleus and EDL, respectively (Fig. 6C) and also
reduced FoxO1Ser256 phosphorylation in both muscles (Fig. 6D). Cold exposure for 24 h also led to a decrease in 4E-BP1 phosphorylation by 40% in soleus and 65% in EDL muscle (Fig. 2B). The phosphorylation status of AMPK172 was increased by 64% in EDL, but not in soleus, from rats exposed to cold (Fig. 6B).

Effect of cold exposure on expression of μ-calpain, calpastatin, and fodrin cleavage. To further investigate the effect of cold on the Ca²⁺-dependent system, mRNA levels and protein content of μ-calpain and calpastatin were measured. Because removal of the NH₂ terminus region of the 80-kDa catalytic subunit of calpains by autolysis can be used as a marker of calpain activation (23), we also assessed μ-calpain autolysis in the soluble fraction of our extracts. Exposure to cold decreased mRNA levels of μ-calpain (Fig. 7A) and increased the autolysed form of μ-calpain (Fig. 7B), but it did not affect mRNA levels (Fig. 8B) or protein content (Fig. 8C) of calpastatin in EDL muscles after 24 h. Although the Ca²⁺-dependent activity estimated in isolated soleus muscles was not altered, exposure to cold increased both the gene expression (Fig. 7A) and the autolysed form of μ-calpain (Fig. 7B). This effect was associated with an increase in calpastatin protein levels after 12 h of cold exposure (Fig. 8A), whereas gene expression (Fig. 8B) and protein content (Fig. 8C) of calpastatin remained unchanged after 24 h of cold exposure. The 145-kDa fragment generated by calpain cleavage of fodrin was increased 82% only in EDL of rats exposed to cold for 24 h, with no changes observed in soleus muscles (Fig. 7C).

No changes were observed in μ-calpain protein content after 24 h of cold exposure either in soleus or in EDL muscles (data not shown).

DISCUSSION

The present findings provide new insights into the mechanisms and signaling pathways through which acute cold stress exerts deleterious effects on muscle protein metabolism. In addition to confirming that short-term exposure to cold decreases the rate of muscle protein synthesis (Fig. 2A), the present work shows for the first time that the rate of protein degradation acutely increases in both oxidative (soleus) and glycolytic (EDL) skeletal muscles of rats (Fig. 1, A and B). Indeed, within hours of cold exposure onset (i.e., 6 h in soleus; 12 h in EDL), the rate of muscle protein degradation increased by as much as 20%. The fall in the rate of protein synthesis and rise in proteolytic rate observed herein during the first 24 h of exposure to cold occurred simultaneously with the well-known sympathetic activation, which led to a large increase in plasma levels of glucose, corticosterone and thyroid hormones; and a decrease in plasma insulin (Table 1). Therefore, all these endocrine changes may have accounted for the cold-induced catabolic effects on muscle protein metabolism, and in this model of stress it is hard to discriminate which hormonal changes are primarily responsible for the effects observed here.

There is little published literature to compare our results, and the reported effects of cold stress on muscle protein turnover have been contradictory. A number of studies utilizing radioisotopically labeled amino acids have reported decreases in the fractional synthesis rate with no alteration in protein degradation in skeletal muscles of young male rats exposed to cold for 2 or 5 days (26, 34). However, other studies have found increases in both protein synthesis and protein degradation in adult female rats exposed to cold for 7 days (7). McAllister et al. (26) investigated the effect of long-term cold exposure (20 days) on skeletal muscle protein turnover in young growing rats and concluded that long-term cold acclimation in skeletal muscle is associated with the establishment of a new steady state in protein turnover with decreased protein mass and normal fractional rates of protein turnover. These conflicting results among studies could be attributed to differences in the age of animals studied, given that compared with adult muscles, young growing muscles are more responsive to growth stimuli and have a higher capacity to activate molecules that promote protein translation (38). However, differences in the technique used for measuring protein metabolism and in the experimental design, such as duration and intensity of cold, and timing of measurements, cannot be ruled out.

Our data clearly demonstrate that the lysosomal proteolytic system does not participate in any of the changes in overall skeletal muscle proteolysis induced by cold exposure, because the activity of this system remained unchanged in soleus and EDL throughout the experimental period (Fig. 4, A and B). On the other hand, the increase in the rate of overall proteolysis (detected during the first 24 h) was accompanied by a parallel increase in UPS activity in both soleus and EDL muscles (Fig. 4). Activity of lysosomal, calcium-dependent, and ubiquitin (Ub)-proteasome proteolytic systems in soleus (A) and EDL (B) muscles of rats exposed to cold for 24 h. The control group was considered to be 100%. Values are means ± SE of 7 rats for each condition; *P < 0.05 vs. control. Control values (nmol·mg⁻¹·h⁻¹) for soleus are 0.071 ± 0.008, 0.065 ± 0.010, and 0.121 ± 0.011; and for EDL they are 0.148 ± 0.013, 0.049 ± 0.004, and 0.078 ± 0.005 for the lysosomal, calcium-, and Ub-proteasome proteolytic systems, respectively.

Fig. 4. Activity of lysosomal, calcium-dependent, and ubiquitin (Ub)-proteasome proteolytic systems in soleus (A) and EDL (B) muscles of rats exposed to cold for 24 h. The control group was considered to be 100%. Values are means ± SE of 7 rats for each condition; *P < 0.05 vs. control. Control values (nmol·mg⁻¹·h⁻¹) for soleus are 0.071 ± 0.008, 0.065 ± 0.010, and 0.121 ± 0.011; and for EDL they are 0.148 ± 0.013, 0.049 ± 0.004, and 0.078 ± 0.005 for the lysosomal, calcium-, and Ub-proteasome proteolytic systems, respectively.
This increase was probably a consequence of the increase in the ubiquitination process, because the protein and mRNA levels of atrogin-1 and MuRF-1 (Fig. 5) were drastically upregulated in muscles from cold-exposed rats. In agreement with these data, one cellular signaling pathway that controls Ub-proteasome-dependent protein breakdown was hindered. Specifically, we found a decrease in pAkt (Fig. 6A) and pFoxO (Fig. 6, C and D) in both soleus and EDL muscles under acute cold stress. The Akt-dependent pathway is not only involved in inhibition of proteolysis, but it has also been implicated in stimulation of signaling pathways responsible for muscle protein synthesis (5). The finding of the present study that cold exposure decreased phosphorylation levels of the eukaryotic Initiation Factor 4E Binding Protein 1, 4E-BP1 (Fig. 2B), a downstream target of mammalian target of rapamycin (mTOR), which is activated by Akt (32), is consistent with the low rate of protein synthesis found in both soleus and EDL muscles of young growing rats (Fig. 2A). Altogether, these results provide evidence that short-term cold exposure is associated with a reduction in protein synthesis and activation of the UPS, and atrogen expression in fast- and slow-twitch skeletal muscles, which may be consistent with impaired Akt signaling. AMPK is a sensor of energy status that maintains cellular energy homeostasis and, when phosphorylated, may inhibit protein synthesis by, at least in part, decreasing 4E-BP1 phosphorylation (6, 41) and may activate expression of atrogens (20, 47) through enhancing the activity of the FoxO family of transcriptional factors (16). Interestingly, we have observed the greater cold-stress AMPK phosphorylation (activation) exclusively in EDL (Fig. 6B), suggesting that the AMPK pathway may also mediate the deleterious effect of cold stress on protein metabolism in fast-twitch muscles. It is noteworthy that rats did not lose muscle mass after 24 h of cold exposure, even with a decrease in protein synthesis and an
increase in UPS activity and atrogene expression observed in these animals. However, when animals were exposed to an extended period of cold (4 days), the loss of mass was observed in soleus, tibialis, and gastrocnemius (Fig. 3B), supporting the hypothesis that protein metabolism alterations we observed during the first 24 h are the preamble of muscle atrophy in continuously cold-exposed animals. Therefore, the length of cold exposure is an important factor for muscle atrophy in young growing rats.

The cold-induced endocrine changes, including low levels of insulin and/or increased levels of corticosterone, could cause impairment in Akt/FoxO signaling, leading to activation of atrogene and accelerated muscle protein degradation by proteasome. Wang et al. (49) have shown that insulin resistance causes muscle protein degradation by mechanisms that involve suppression of phosphatidilinositol-3-kinase/Akt signaling, leading to activation of UPS. Moreover, a high physiological amount of glucocorticoid can stimulate expression of atrogin-1 and MuRF1 by decreasing phosphorylation of FoxO (22, 35). Hu et al. (17) reported a nongenomic action of the glucocorticoid that consists of impairing the association of the insulin receptor substrate 1 (IRS-1) with PI3K, leading to a decrease in Akt phosphorylation. High levels of thyroid hormones have been shown to increase UPS activity, and protein and mRNA contents of atrogin-1 and MuRF1 in rat EDL muscles (28). Future studies should reveal the extent to which these different mechanisms contribute either alone or in combination to activation of the UPS in short-term cold-exposed animals.

In addition to changes observed in the UPS, the great increase in the Ca2+-dependent proteolytic system (estimated by the high proteolytic capacity and autolysed form of calpain with no changes in calpastatin) also contributed to the increase in overall protein degradation only in EDL muscle from cold-exposed rats. In agreement with this finding, the 145-kDa
α-fodrin fragment, which is an indicator of calpain activity, was increased in EDL muscle from rats exposed to cold (Fig. 7C). Despite these changes, the rats submitted to cold presented a decrease in α-calpain mRNA in EDL (Fig. 7A). This decrease can be interpreted as a restoration of muscle capacity to limit protein breakdown by calpains, although the underlying mechanisms cannot be explained on the basis of the present data. Unlike EDL, soleus muscle did not show any change in Ca²⁺-dependent proteolysis in cold-exposed rats (Fig. 4A). This fact could be explained at least in part by the cold-induced increase in calpastatin levels (Fig. 8A), which could inhibit the proteolytic action of α-calpain despite the increase in its expression and protein content during cold exposure (Fig. 7, A and B) (24). In fact, no change in the 145-kDa fragment product of α-fodrin cleavage was detected between groups. This increase in calpastatin protein levels may be due to the enhanced sympathetic activity induced by cold, which is supported by the findings that activity and gene expression of calpastatin are increased after epinephrine (29) or β₂-adrenergic agonist treatment in rat soleus muscle (15). We cannot exclude the possibility that the sympathetic activation in this model of cold exposure could have a sparing effect on protein metabolism, limiting the rise of protein degradation (2).

In summary, the present data suggest that short-term cold exposure for 24 h promoted a decrease in the rate of protein synthesis and an increase in the activity of UPS in both muscles studied as well as in the expression of atrophy-related genes (atrogin-1 and MuRF1) in skeletal muscles of young rats. Ca²⁺-dependent proteolytic activity was greatly increased only in EDL muscle. The activation of atrophy induced by acute cold stress seems to be mediated through the inactivation of Akt/FoxO signaling, and possibly activation of AMPK. We speculate that these changes in muscle protein metabolism
during the first hours of cold exposure are physiologically important to increase circulating free amino acids, which can be used as fuel for heat production and/or for gluconeogenesis.

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AUTHOR CONTRIBUTIONS


DISCLOSURES

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